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RAD51C – a new human cancer susceptibility gene for sporadic squamous cell carcinoma of the head and neck (HNSCC)

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Abstract

Introduction—Head and neck squamous cell carcinomas (HNSSCs) are one of the leading causes of cancer-associated death worldwide. Although certain behavioral risk factors are well recognized as tumor promoting, there is very little known about the presence of predisposing germline mutations in HNSCC patients.

Methods—In this study, we analyzed 121 individuals with HNSCCs collected at our institution for germline alterations in the newly identified cancer susceptibility gene *RAD51C*.

Results—Sequencing of all exons and the adjacent introns revealed five distinct heterozygous sequence deviations in *RAD51C* in seven patients (5.8%). A female patient without any other risk factors carried a germline mutation that disrupted the canonical splice acceptor site of exon 5 (c. 706-2A>G).

Conclusions—As there are only a few publications in the literature identifying germline mutations in head and neck cancer patients, our results provide the first indication that paralogs of RAD51, recently implemented in breast and ovarian cancers, might also be candidates for genetic risk factors in sporadic squamous cell carcinomas of the head and neck.

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Conflict of interest statement

Kathrin Scheckenbach, Marcel Freund and Helmut Hanenberg may receive royalties based on a licensing agreement with Myriad Genetics for the use of RAD51C as a cancer susceptibility gene. All other authors declare to have no conflict of interest.

Introduction

Six percent of all human malignant tumors worldwide are squamous cell carcinomas of the head and neck region (HNSCCs) (1). In Europe, the consumption of alcohol and nicotine are the main behavioral risk factors to develop HNSCC (2, 3), however infections with the oncogenic human papilloma virus (HPV) −16 or −18 strains also contribute significantly to tumorigenesis and incidence of HNSCCs (4). Genetic factors might also play a role in the development of HNSCCs at a relatively early age and especially in the absence of any known tumorigenic trigger, however almost all changes described were somatic mutations present in the tumor cells (5–13).

Fanconi anemia (FA) is a rare inherited recessive disorder usually diagnosed around 10 years of age, where patients without behavioral risk factors frequently develop HNSCCs in their early adulthood (14–17). The genetic causes for FA are autosomal or X-chromosomal germline defects in at least 16 different genes involved in the repair of DNA crosslinks at stalled replication forks (18–22). Cells with defects in the FA pathway show spontaneous chromosomal instability and a characteristic hypersensitivity to DNA crosslinking agents such as mitomycin C (MMC) (23). Clinically, FA patients are characterized by congenital abnormalities, progressive bone marrow failure, and the predisposition to leukemia and epithelial cancers (15). Remarkably, about 50% of FA patients without stem cell transplantation and nearly 100% of transplanted FA patients develop a squamous cell carcinoma of the head and neck until 45 years of age (17).

During the last six years, heterozygous germline and also acquired defects of FA genes were identified in patients with sporadic epithelial cancers such as breast (24–26), ovarian (27, 28), cervical (29), lung (30), pancreatic (31, 32) or testicular cancer (33). Since genetic instability is also seen as a major force for driving head and neck cancer tumorigenesis (34), heterozygous germline defects in genes of the FA pathway could also be a predisposing genetic condition for the development of HNSCC. Indeed, initial work identified defects in the FA pathway in sporadic HNSCCs, such as downregulation of several FA genes in sporadic HNSCCs (35), inactivating promoter methylation in *FANCB* (36) and defective FANCD2 foci formation after stimulation (37).

Recently, we identified *RAD51C* (*RAD51L2*), putatively designated as *FANCO* (38), as a human cancer susceptibility gene for inherited breast and ovarian malignancies (39). RAD51C is one of five paralogues (RAD51B, RAD51C, RAD51D, XRCC2, XRCC3) of the highly conserved RAD51 recombinase that plays a central role in the homologous repair (HR) of DNA double strand breaks (DSBs) in mammalian cells, mediating homologous DNA pairing and strand exchange (40, 41). In cells that are defective in any of the paralogues, the formation of RAD51 foci is strongly reduced in response to DNA damage and correlates with decreased HR efficiency, increased genomic instability and a higher incidence of chromosomal abnormalities (41, 42).

In order to determine whether *RAD51C* germline mutations are also a predisposing condition for the development of HNSCC, peripheral blood (PB) DNA from 121 patients

(male: 97; 80.2%, female: 24; 19.8%) diagnosed with sporadic HNSCCs at our institution was analyzed for *RAD51C* mutations by direct sequencing (38, 39).

Materials and Methods

Patients

PB samples were collected from 121 consecutive patients of the Department of Otorhinolaryngology (Heinrich-Heine University, Düsseldorf, Germany) with histologically confirmed HNSCCs, including all sites (55 oropharynx, 20 hypopharynx, 27 larynx, 7 Sinus, 2 scalp, 10 CUP (carcinoma of unknown primary)) and stages (cis, $T_{1–4}$, $N_{0–3}$, $M_{0/1}$) of disease after obtaining informed consent. Research was carried out in compliance with the Helsinki Declaration. This study was reviewed and approved by the ethics committee of the University of Düsseldorf (Study No. 3515).

HPV Status

For immunohistochemical staining of p16/INK4a, 2-μm sections were stained using the CINtec PLUS kit (mtm Laboratories, Heidelberg, Germany) according to the manufacturer's instructions as described previously (43).

Sequencing of all 9 exons of the RAD51C gene

DNA was isolated from PB leukocytes according to the manufacturer's recommendation (Genomic DNA purification kit, Gentra Biosystems, Minneapolis, USA). Each of the 9 exons of the *RAD51C* gene was amplified in a standard PCR reaction using Qiagen Mastermix (Qiagen, Hilden, Germany). Primers and PCR conditions are shown in Table 1. The PCR products were purified (Qiaquick PCR Purification Kit, Qiagen), and mixed with ABI PRISM BigDye Terminator sequencing kit (Applied Biosystems, Weiterstadt, Germany) and primers for sense direction or for antisense direction (Table 1). After the sequencing reaction (25 cycles of 15 sec at 96°C and 4 min at 60°C), the products were purified (DyeEx 2.0 Spin Kit, Qiagen) and analyzed with an automated sequencer (ABI 310, Applied Biosystems). Positive samples underwent confirmation by repeated analysis.

Results

In total, we identified five heterozygous germline alterations in seven (3 females, 4 males) out of 121 individuals. All sequence changes were single nucleotide alterations (Table 2). The mutation c.706-2A>G was located in the canonical splice acceptor dinucleotide of intron 4 in the germline DNA from a female without any behavioral risk factors and is clearly pathogenic as it leads to a loss of exon 5 in the mRNA resulting in an in-frame deletion of 44 amino acids, pV236del44, in the RAD51C protein. This germline mutation was previously described by Walsh *et al*. 2011 (44) in a 70-year old stage IV ovarian cancer patient with loss-of-heterozygosity (LOH) in the tumor tissue and by Loveday *et al*. 2012 (45) in a 56-year old female with ovarian cancer. Four missense alterations identified in six patients were located in the exons 1 (1×, c.7G>A, p.G3R), 2 (2×, c.376G>A, p.A126T), 5 (1×, c.790G>A, p.G264S) and 6 (2×, c.859A>G, p.287A), respectively.

Four of the HNSCCs were located at the larynx, two in the oropharynx and one in the hypopharynx. The two patients with laryngeal carcinomas developed additional malignancies: one experienced an oropharyngeal carcinoma and one was diagnosed with bronchial carcinoma (Table 2). As overexpression of the p16 protein can be used as a surrogate marker for HPV infection in tissues (45), we analyzed the available tumor tissue samples of the seven patients by immunohistochemistry: samples from three patients stained p16 positive, suggesting HPV infections, and three samples were negative. From one patient (#5), tumor tissue was not available. Two patients had a combined history of regular alcohol uptake and cigarettes consumption, two other individuals smoke cigarettes, while three patients were not exposed to any of these drugs (Table 2).

Discussion

We identified five *RAD51C* germline alterations in seven patients with sporadic HNSCC, representing 5.8% of all analyzed 121 individuals. All patients who carried *RAD51C* alterations had tumors that originate from the mucus membrane as most frequent carcinoma type in our series. Four of the five changes were missense alterations that we previously reported in individuals from German pedigrees with breast and ovarian cancers (39). The functional characterizations of these four RAD51C alterations in this earlier study revealed that expression of c.7G>A (patient 1) and c.376G>A (patients 2 and 3) mutated *RAD51C* cDNAs transferred via retroviral vectors in *Rad51c*−/− chicken DT40 cells and in human *RAD51C*-mutated fibroblasts was associated with normal cellular survival and normal RAD51 foci formation in response to MMC exposure, respectively (39). In contrast, expression of the c.790G>A (patient 5) and the c.859A>G (patient 6 and 7) *RAD51C* alterations showed clearly reduced survival of *RAD51c*−/− DT40 cells upon MMC challenge albeit normal RAD51 foci formation (39). As the latter two missense alterations were associated with impaired cellular viability in response to DNA cross-linking agent MMC (39), we had hypothesized that these two alterations should therefore be associated with an increased cancer risk. However, comparative analysis with 2912 representative control individuals of the German population revealed that only the c.790G>A alteration in *RAD51C* was associated with an increased risk of 3.44 (confidence interval 1.51–7.8, $p<0.005$) for developing gynecological cancers (39). A similar association of *RAD51C* c.790G>A mutation with an increased cancer risk was also confirmed in the British study by Loveday *et al*. (45). The fifth *RAD51C* germline alteration in our patient population was a splice acceptor mutation, c.706-2A>G, that disrupts the canonical AG splice acceptor site and thereby leads to a loss of exon 5 in the transcript and a frameshift in the open reading frame with premature protein truncation. This mutation was previously detected in two individuals with ovarian cancer and classified in both publications as detrimental (44, 45).

Strikingly, three out of the seven HNSCC patients with germline alterations in *RAD51C* were females (43%), while only 20% of all patients in our patient cohort were women. All three women did not have any history of tobacco and alcohol consumption as typical triggers for the development of HNSCCs. The tumor in the patient 3 with the functionally normal c.376G>A alteration (39) was classified as HPV positive, thus providing an explanation for the HNSCC development in this individual. Importantly, the absence of any risk factor in patient 4 (c.706-2A>G) with a HPV16/18 negative tumor and the development

of an independent lung cancer in patient 5 (c.790G>A) strongly indicated an underlying genetic cancer susceptibility in these two women, likely due to the *RAD51C* germline mutations. In contrast, all four male patients had a history of smoking cigarettes. Two also consumed alcohol and their tumors were classified as HPV positive. Therefore, the risk factor profiles in these four individuals were similar to those of patients without any alterations in *RAD51C* and characteristic for HNSCC patients. Hence, an association of the development of HNSCCs with germline changes in *RAD51C* was not evident in the four male patients.

The most important risk factors for HNSCCs in the western hemisphere are behavioral factors, predominantly chronic exposure to tobacco and/or alcohol and infection with HPV (4). HNSCC cancer carcinogenesis has been linked to abnormalities in DNA repair, apoptosis, carcinogen metabolism and cell cycle control (1) and somatic changes in associated genes in HNSCC tissues were described by several groups (5, 7, 8, 47–49). However, specific germline mutations predisposing to HNSCCs have only been identified in *P53* and *INK4a/p16* that are associated with a wide variety of human cancers (50, 51). Here, we provide first evidence that germline alterations in the established cancer susceptibility gene *RAD51C* (38, 39, 44, 45) are also present in HNSCC patients. These alterations in the RAD51C protein in at least 4 out of 121 patients (3%) are associated with reduced (patients 5, 6 and 7) (39) or absent (patient 4, c.706-2A>G) function, thus suggesting that germline mutations in genes of the FA pathway could contribute to the development of HNSCCs. Here, further studies of analyzing HNSCC patients for germline mutations in FA-associated genes will provide further insides whether the ’common disease, rare allele’ hypothesis (52) might also apply to a subset of HNSCC patients at relatively young age and without known risk factors.

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Table 1

Exon	Forward 5’ to 3’	Reverse 3’ to 5’	Tm (°C)	Size (bp)
1	AAATGGGATTTTGGGGAATC	GTAAACATGGACGTGGGAGG	TD*	471
2	AAAATTAAATGGTTGATAGAATGTTGC	TCAAGAAGGGATAATGAAGTAACAC	65	583
3	GACATTTCTGTTGCCTTGGG	GCTGTGGCATTTCTCATTTTG	65	472
4	TTTTGCTATAATTTGTCATCTTTCAG	TTGTAGGTCAAGGAAGGAAGAGA	60	413
5	TTACTGTTCCAGGCATTGGG	TGGAAACCAACCAAACGTAAC	65	430
6	GTGCATGCCACCATGTCT	TGTGTCTGGCCACTCAATAAA	68	398
7	GAATAATGATTTGCAGTATTTCC	CAGACAAGGCAACAAAAGTGTC	65	400
8	CATACGGGTAATTTGAAGGGTG	TTTGGGGACAATGTTCTAAGC	65	384
9	CGCCTGGCCCTAGAATAAA	GGCCACATGAGATCAGCTTT	65	491

* Primer sequences used to amplify and sequence RAD51C, with annealing temperature (Tm) and amplicon size (TD – touchdown PCR, indicates that DMSO was added to a final concentration of 10%).

Table 2

Patient	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Gender	M	M	F	F	F	M	M
Location	Exon 1	Exon 2	Exon 2	Intron 4	Exon 5	Exon 6	Exon 6
Nucleotide change	c.7G>A	c.376G>A	c.376G>A	c.706–2A>G	c.790G>A	c.859A>G	c.859A>G
Protein change	p.G3R	p.126A>T	p.126A>T	Aberrant splicing	p.264G>S	p.287T>A	p.287T>A
Tumor localization	Larynx	Larynx	Oropharynx	Oropharynxnx	Larynx	Hypopharynx	Larynx
TNM	pT2pN2M0	pT1bN0M0	pT4apN0M0	pT3pN1M1	pT2pN2bM0	pT2N0M0	pT4apN0M0
Additional cancers	Oropharynx (pT3pN0M0)	-	-	-	Broncial CA (T3N2bM0)	-	-
Nicotine	Y	Y	N	N	N	Y	Y
Alcohol consumption	Y	N	N	N	N	N	Y
HPV	positive	negative	positive	negative	n.t.	negative	positive
Detected in breast cancer	Y	Y	Y	N (ovarian)	Y	Y	Y
Functionally tested	Y	Y	Y	N	Y	Y	Y
Detrimental mutation	N	N	N	Y	Y	Y	Y
Appearance in dbSNP	N	Y	Y	N	Y	Y	Y
dbSNP number	n/a	rs61758784	rs61758784	n/a	rs147241704	rs28363317	rs28363317
Allele count (MAF source: 1000 Genomes)*	n/a	A = 0.001/2	A = 0.001/2	n/a	A = 0.002*	G = 0.006/13	G = 0.006/13

Y = yes; n = no; n.t.= not tested (no specimen available); F = female; M = male; Ex = Exon; MAF = minor allele frequency;

*source = University of Washington/Broad Institute; functional testing as depicted in Meindl et al. [39].